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# Sealing of Chromosomal DNA Nicks during Nucleotide Excision Repair Requires XRCC1 and DNA Ligase III $\alpha$ in a Cell-Cycle-Specific Manner

Jill Moser,<sup>1</sup> Hanneke Kool,<sup>1</sup> Ioannis Giakzidis,<sup>2</sup> Keith Caldecott,<sup>2</sup> Leon H.F. Mullenders,<sup>1,\*</sup> and Maria I. Fousteri<sup>1,\*</sup>

<sup>1</sup>Department of Toxicogenetics, Leiden University Medical Center, Einthovenweg 20, Leiden 2333 RC, The Netherlands

<sup>2</sup>Genome Damage and Stability Center, University of Sussex, Science Park Road, Falmer, Brighton BN1 9RQ, UK

\*Correspondence: [l.mullenders@lumc.nl](mailto:l.mullenders@lumc.nl) (L.H.F.M.), [m.i.fousteri@lumc.nl](mailto:m.i.fousteri@lumc.nl) (M.I.F.)

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## SUMMARY

Impaired gap filling and sealing of chromosomal DNA in nucleotide excision repair (NER) leads to genome instability. XRCC1-DNA ligase III $\alpha$  (XRCC1-Lig3) plays a central role in the repair of DNA single-strand breaks but has never been implicated in NER. Here we show that XRCC1-Lig3 is indispensable for ligation of NER-induced breaks and repair of UV lesions in quiescent cells. Furthermore, our results demonstrate that two distinct complexes differentially carry out gap filling in NER. XRCC1-Lig3 and DNA polymerase  $\delta$  colocalize and interact with NER components in a UV- and incision-dependent manner throughout the cell cycle. In contrast, DNA ligase I and DNA polymerase  $\epsilon$  are recruited to UV-damage sites only in proliferating cells. This study reveals an unexpected and key role for XRCC1-Lig3 in maintenance of genomic integrity by NER in both dividing and nondividing cells and provides evidence for cell-cycle regulation of NER-mediated repair synthesis in vivo.

## INTRODUCTION

Nucleotide excision repair (NER) is a multistep process that removes a wide variety of helix-distorting DNA lesions by a dual incision mechanism. Global genome repair (GGR) is the NER subpathway that deals with genome-wide repair of lesions. Defects in genes encoding for GGR proteins give rise to the UV-sensitive and cancer-prone genetic disorder xeroderma pigmentosum (XP) (Friedberg, 1996). GGR is subdivided into pre- and postincision stages. The preincision stage has been studied extensively in vivo and in vitro and comprises the step of DNA damage recognition, local unwinding of the DNA and verification of the lesion, and the generation of 3' and 5' incisions flanking the DNA lesion (reviewed in Gillet and Scharer [2006]). The postincision stage of NER involves gap filling by DNA repair synthesis, ligation, and restoration of chromatin

structure; however, its regulation and the factors involved in vivo are much less understood. Reconstituted mammalian NER systems demonstrated that DNA repair synthesis could be executed by both replicative DNA polymerases  $\delta$  and  $\epsilon$  (pol $\delta$  and pol $\epsilon$ ) in the presence of RPA, RFC, and PCNA. In these studies, DNA ligase I (Lig1) was able to seal the nick in the DNA preferably in combination with pol $\epsilon$  (Aboussekhra et al., 1995; Shivji et al., 1995).

Lig1 has an essential role in DNA replication and has been implicated in long-patch base excision repair (BER) and NER (reviewed in Tomkinson and Levin [1997] and Martin and MacNeill [2002]). The involvement of Lig1 in NER in vivo was primarily based on the UV sensitivity of cells derived from a patient deficient in Lig1 (46BR [Teo et al., 1983]). 46BR cells contain normal levels of Lig1 protein but display only ~3%–5% DNA joining activity due to mutations in the catalytic domain of the protein (Barnes et al., 1992). Biochemical studies showed that more strand breaks persisted in UV-irradiated 46BR than in normal cells (Nocentini, 1995). However, additional studies with 46BR cells did not show enhanced UV sensitivity (Bentley et al., 2002); moreover, wild-type and Lig1 mutant mouse fibroblasts displayed no significant differences in sensitivity to UV (Bentley et al., 2002).

In addition to Lig1, two other mammalian DNA ligases (III and IV) have been described that target to different repair and replication pathways (Martin and MacNeill, 2002). DNA ligase IV (Lig4) and its partner protein, XRCC4, are involved in repair of DNA double-strand breaks (DSBs) by nonhomologous end joining (NHEJ) and V(D)J recombination (Grawunder et al., 1998). DNA ligase III $\alpha$  (in the following referred to as Lig3) and its partner protein, XRCC1, are key components of both single-strand break repair (SSBR) and BER (Caldecott, 2003; Brem and Hall, 2005). XRCC1 and Lig3 interaction is mediated by their C termini, both of which encode for a BRCT domain (Nash et al., 1997; Taylor et al., 1998).

Herein we describe the identification of two unanticipated factors required for the ligation step in NER. We show for the first time, to our knowledge, that XRCC1 and Lig3 are essential core components of mammalian NER. Our data demonstrate that downregulation of Lig3 impairs removal of UV lesions and rejoining of UV-induced nicks in chromosomal DNA. Moreover, XRCC1-Lig3

together with pol $\delta$  interacts and colocalizes with NER components in a UV-specific manner throughout interphase. On the other hand, recruitment of Lig1 and pol $\epsilon$  to UV-irradiated sites is only observed in proliferating cells, suggesting a differential requirement of DNA ligases and polymerases in NER-mediated repair synthesis during the cell cycle. Based on the above, we discuss the role of the newly identified components in NER and the likely mechanisms by which NER is regulated in mammalian cells.

## RESULTS

### Lig1-Deficient Cells Exhibit Efficient Repair of UV Photolesions

To study the regulation of the postincision mechanism of NER in vivo, we initially focused on the ligation of the NER-mediated repair patches. Evidence for a functional role of Lig1 in mammalian NER has been gained from in vitro studies (Aboussekhra et al., 1995; Araujo et al., 2000) and the UV sensitivity of the Lig1 mutant cell line 46BR (Teo et al., 1983). To delineate the role of Lig1 in vivo, we examined the repair of 6-4PP in UV-exposed confluent primary normal human (VH25) and Lig1-deficient fibroblasts (46BR). As controls, we examined Lig4-deficient (SC2) and NER-deficient XP-A cells. Additionally, we employed UV-irradiated confluent normal human cells treated with hydroxyurea (HU) and cytosine- $\beta$ -arabino-furanoside (AraC) to inhibit repair-patch synthesis and ligation (Mullenders et al., 1985; Smith and Okumoto, 1984). Immunofluorescence measurements demonstrated equally efficient levels of repair of 6-4PP in normal human, Lig1-, and Lig4-deficient cells (Figure 1A). In contrast, XP-A and normal cells treated with HU/AraC showed no repair of 6-4PP up to 16 hr after UV irradiation (Figure 1A). We also checked the assembly/disassembly of the NER incision complex in the various cell lines by using local UV irradiation and immunofluorescence (Volker et al., 2001). Normal cells treated with HU/AraC (Figure 1B) displayed retarded disassembly of the NER preincision complex (monitored by XPA or a TFIIH-specific antibody, p89); in contrast, the kinetics of disassembly of the NER preincision complex in confluent Lig1 and two distinct Lig4-deficient cell lines (SC2 and 411BR cells) were similar to normal cells.

We assessed the effect of Lig1 deficiency on repair-patch ligation by using the alkaline comet assay (Figure 1C). We employed normal (VH10/SV) and Lig1-deficient (46BR/SV) SV-40 immortalized cells to allow useful comparison with the siRNA-transfected SV-40 cell lines (Figure 5). In the absence of UV irradiation, the comet tail size of normal cells treated or mock treated with HU/AraC did not differ significantly from the tail of Lig1-deficient cells, suggesting that the inhibitors alone or the absence of Lig1 activity had only marginal effects on the frequency of endogenously induced breaks. In all cells, UV irradiation induced an increase in tail size 30 min after UV irradiation when the majority of lesions are still not re-

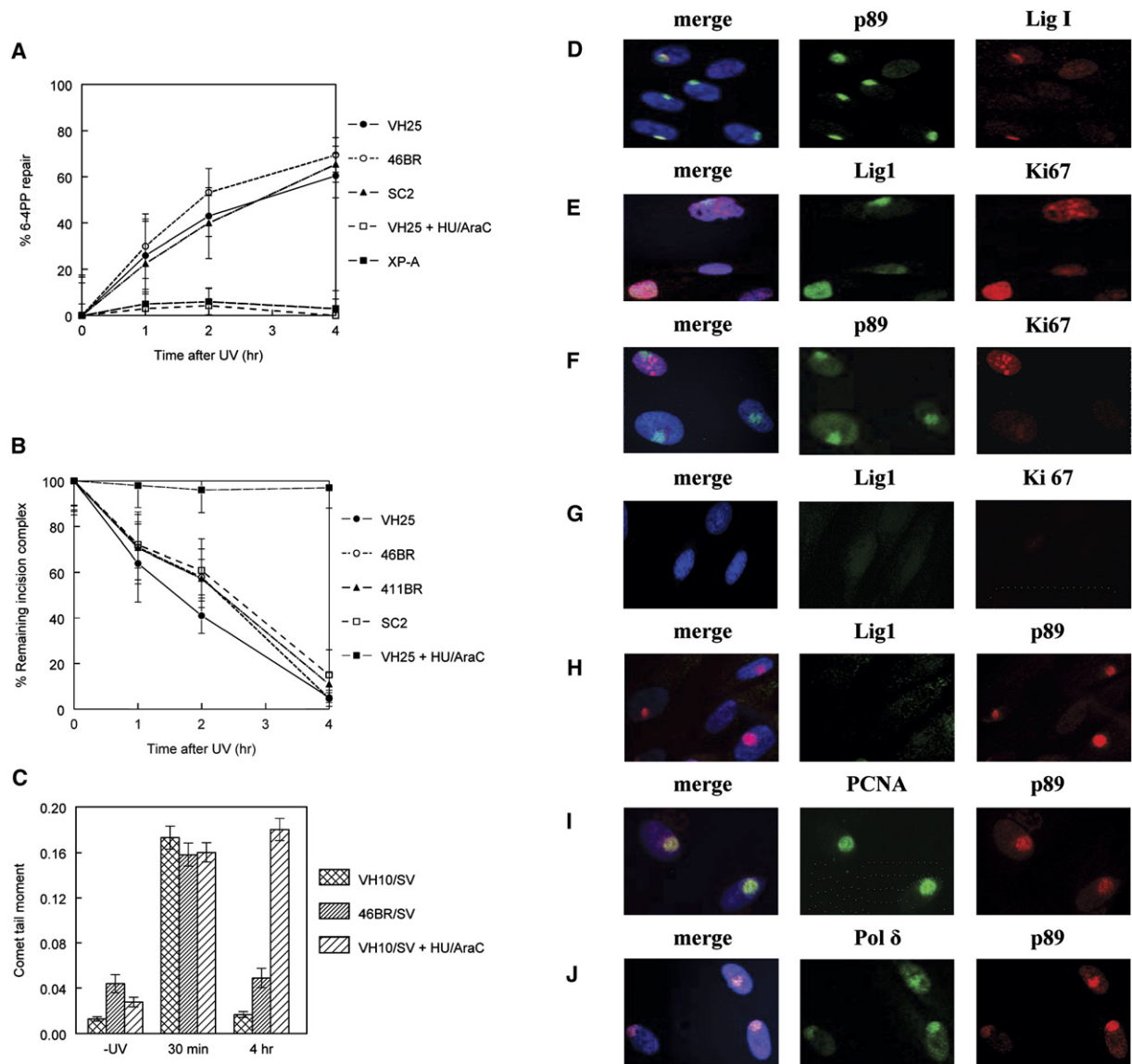
paired. In normal cells treated with HU/AraC, the UV-induced breaks remained open at 4 hr after UV irradiation, whereas normal and Lig1-deficient cells showed efficient sealing of breaks to a level indistinguishable from nonirradiated controls (Figure 1C). Taken together, the above data suggest that Lig1 activity is not essential for the repair of UV photolesions in mammalian cells or sealing the nicks generated during NER.

### Lig1 Colocalizes to Sites of UV-Induced Damage in a Subset of Cells

The efficient repair of UV photolesions in Lig1-deficient human cells and the observation that UV-irradiated wild-type and Lig1 mutant mouse cells display equal levels of clonal survival (Bentley et al., 2002) prompted us to study Lig1 accumulation to UV damage in vivo. We found recruitment of Lig1 to UV spots but surprisingly not in all cells harboring damage: only about 30%–50% of the damaged cells showed Lig1 accumulation (Figure 1D), coinciding with cells that expressed the Ki67 antigen, a marker for proliferation (Figure 1E). The results were identical with various fixation procedures and two different Lig1-specific antibodies, 2B1 and 5H5, recognizing both the unmodified and phosphorylated forms of Lig1 and only the phosphorylated form of Lig1, respectively (Rossi et al., 1999; Vitolo et al., 2005). Lig1 is downregulated in serum-starved (Ki67-negative) human fibroblasts (quiescent cells, G0) when compared to growing human fibroblasts that expressed Ki67 (Vitolo et al., 2005). Local UV irradiation of quiescent cells failed to induce accumulation of Lig1 at sites of UV damage (Figures 1G and 1H). In contrast, preincision NER factors such as p89 (TFIIH, Figure 1F), and the postincision NER components PCNA and pol $\delta$  (also downregulated in quiescent cells) were clearly detectable under these conditions (Figures 1I and 1J). These results show that Lig1 accumulates at UV damage only in proliferating cells.

### NER-Dependent Recruitment of XRCC1 and Lig3 to UV-Induced Damage

We speculated that either Lig3 or Lig4, or both, might perform the ligation step of NER in quiescent cells. However, the normal levels of repair and kinetics of preincision complex disassembly in Lig4-deficient cells (Figure 1A and 1B) together with the lack of recruitment of XRCC4 to UV spots (Figure S1 in the Supplemental Data available with this article online) indicate that Lig4 and its partner protein, XRCC4, are not involved in the repair of UV lesions. Lig3 and its partner, XRCC1, play key roles in BER and SSBR (Caldecott, 2003), but to our knowledge, XRCC1-Lig3 has not been previously implicated in NER. Interestingly, immunostaining of XRCC1 and Lig3 in locally UV-irradiated confluent normal cells revealed localization of both proteins at UV spots 20 min after UV irradiation (Figures 2A and 2B). In fact, both XRCC1 and Lig3 accumulated at UV spots as quickly as 5 min after UV irradiation but were also visible 2 and 4 hr after UV treatment (similar to the other postincision factors, data not shown). In



**Figure 1. DNA Ligase I-Deficient Cells Exhibit Normal Repair of UV-Induced Lesions**

(A) 6-4PP repair as determined by fluorescent immunostaining of 6-4PP at various times after global UV irradiation. Removal (%) of 6-4PP in primary confluent fibroblasts: VH25 (normal), 46BR (Lig1 deficient), and 411BR (Lig4 deficient). Error bars represent the SEM values of 40 nuclei.

(B) Disassembly kinetics of the NER preincision complex. Disassembly was monitored by fluorescent immunostaining of p89 spots at various time points after local UV irradiation in primary confluent fibroblasts. Error bars represent the SEM values of 40 nuclei.

(C) Strand-break repair of globally UV-irradiated (15 J/m<sup>2</sup>) normal and Lig1-deficient cells analyzed by the alkaline comet assay. Breaks were quantified as comet tail moments. Histograms are means  $\pm$  SEM of at least three independent experiments.

Images (D)–(J) were obtained with locally UV-irradiated (30 J/m<sup>2</sup>) primary normal cells. “Merge” refers to the combined image of DAPI and the pair of antibodies used for each panel.

(D) Immunolocalization of Lig1 (2B1 antibody) in confluent cells. UV spots are visualized by local recruitment of TFIIH (p89).

(E) Immunolocalization of Lig1 (5H5 antibody) in proliferating cells as visualized by Ki67 staining.

(F) Immunolocalization of p89 in confluent cells. A proliferating cell is visualized by Ki67 staining.

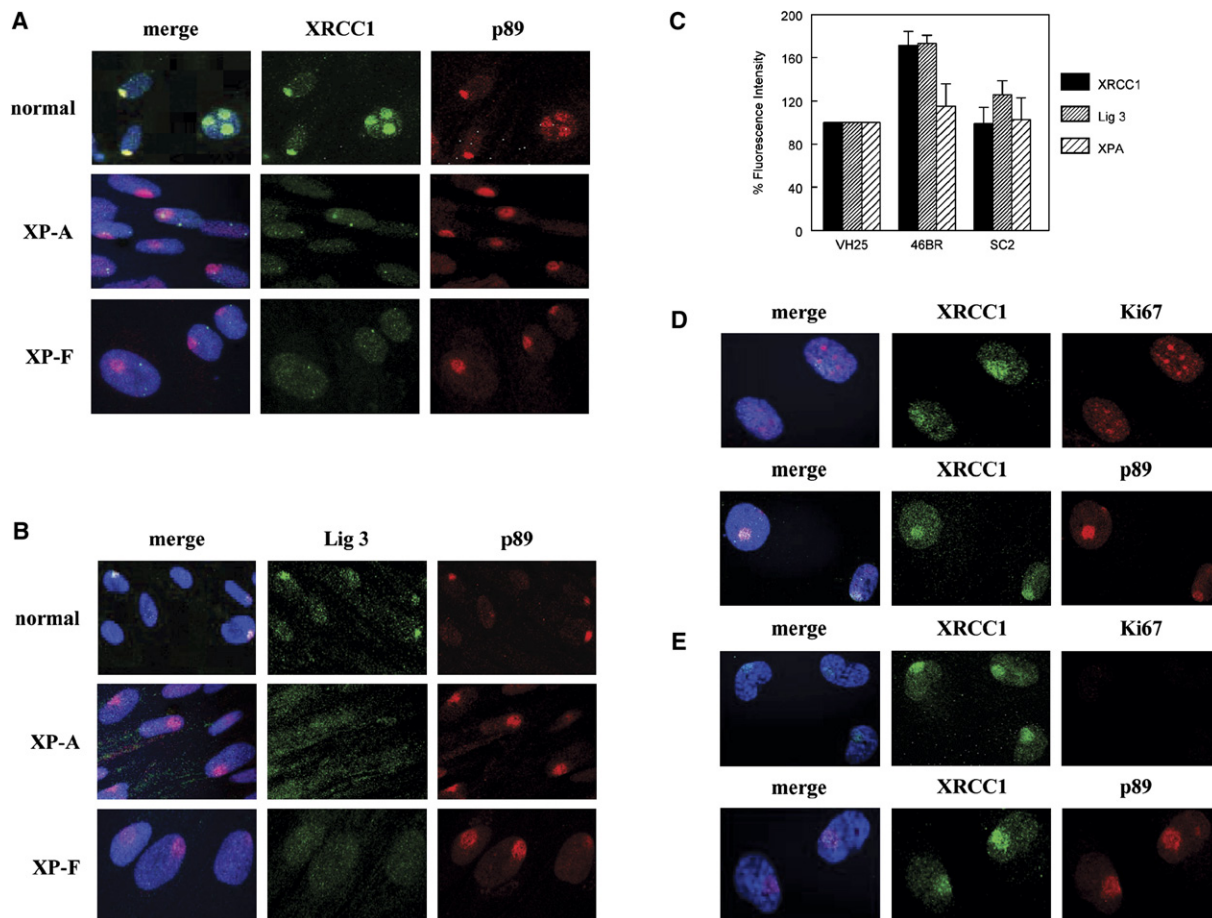
(G) Immunolocalization of Lig1 in quiescent cells as visualized by the absence of Ki67 marker.

(H) Immunolocalization of Lig1 in quiescent cells. UV spots are visualized by recruitment of p89.

(I) Immunolocalization of PCNA and p89 in quiescent cells.

(J) Immunolocalization of DNA pol $\delta$  and p89 in quiescent cells.





**Figure 2. XRCC1 and Lig3 Recruitment to UV-Induced DNA Damage Is Dependent on Functional NER**

(A and B) Immunolocalization of XRCC1 (A) and Lig3 (B) in locally UV-irradiated (30 J/m<sup>2</sup>) confluent normal, XP-A, and XP-F human fibroblasts as indicated. UV-damage sites are visualized by local accumulation of p89.

(C) Relative fluorescence intensity of XRCC1, Lig3, and XPA spots 20 min after local UV irradiation in VH25 (normal), 46BR (Lig1-deficient), and SC2 (Lig4-deficient) cells. Images were taken with equal exposure time. Error bars represent the SEM values of 40 nuclei.

(D) Immunolocalization of XRCC1 and p89 in locally UV-irradiated (30 J/m<sup>2</sup>) cycling cells as visualized by Ki67 staining.

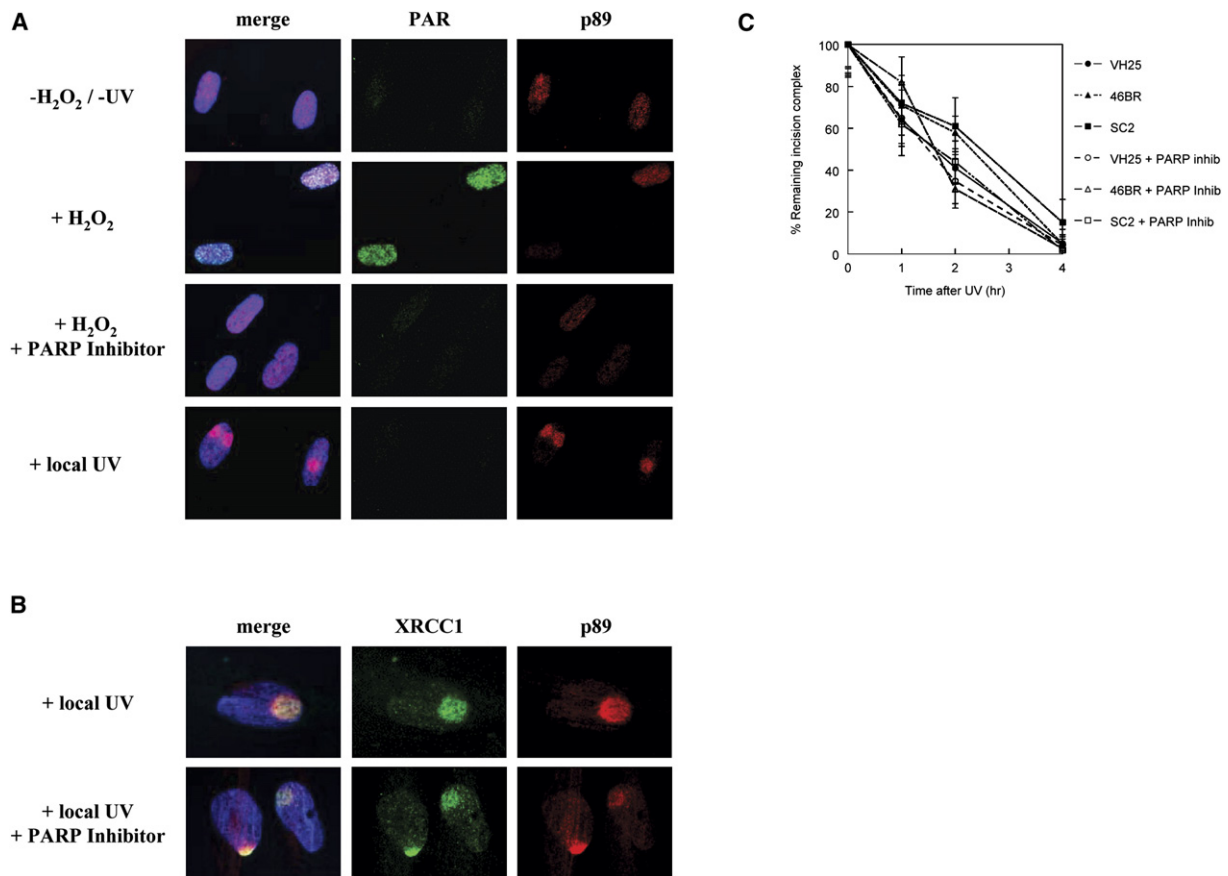
(E) Immunolocalization of XRCC1 and p89 in locally UV-irradiated (30 J/m<sup>2</sup>) quiescent cells as visualized by negative Ki67 staining.

contrast, accumulation of neither XRCC1 nor Lig3 was detected in UV-irradiated confluent XP-A and XP-F cells (Figures 2A and 2B), indicating that recruitment of XRCC1 and Lig3 requires preincision complex assembly. The intensities of XRCC1 and Lig3 at UV spots were ~1.7-fold increased in Lig1-deficient cells when compared to normal and Lig4-deficient cells (Figure 2C). Western blot analysis confirmed the enhanced level of Lig3 in Lig1-deficient cells (Figure 5A). Together these data suggest that XRCC1 and Lig3 are recruited to UV damage in normal human cells in an incision-dependent manner. This event is particularly marked in Lig1-deficient cells, suggesting that Lig3 might compensate for Lig1 deficiency.

XRCC1 and Lig3 accumulated at UV damage and colocalized with p89 in both cycling and quiescent cells (Figures 2D and 2E). These results point to an XRCC1-Lig3-dependent ligation mechanism that functions in mammalian NER in response to UV damage independent

of cell cycle. In contrast, a role for Lig1 in NER-mediated ligation appears to be restricted to Ki67-positive S- or late G1/S phase cells (Figures 1D, 1G, and 1H).

Despite the fact that XRCC1 has no known catalytic activity itself, it does interact with several other DNA repair proteins required for BER and SSB, including poly (ADP-ribose) polymerase 1 (PARP-1) and DNA polymerase  $\beta$  (pol $\beta$ ) (Caldecott, 2003). PARP-1, an enzyme synthesizing poly (ADP-ribose) (PAR) from NAD<sup>+</sup>, is essential to recruit XRCC1 and Lig3 at sites of single-strand breaks (SSBs) (Okano et al., 2003) and oxidative damage (El-Khamisy et al., 2003). To disclose a role of PARP-1 in NER and in the recruitment of XRCC1-Lig3 to UV damage, we performed immunostaining of PAR in primary quiescent normal human fibroblasts exposed either to 100  $\mu$ M hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) for 20 min or to local UV irradiation (Figure 3A). H<sub>2</sub>O<sub>2</sub> exposure induced a strong and homogeneous PAR nuclear staining consistent with previous



**Figure 3. PARP-1-Independent Recruitment of XRCC1-Lig3 to UV Damage Sites**

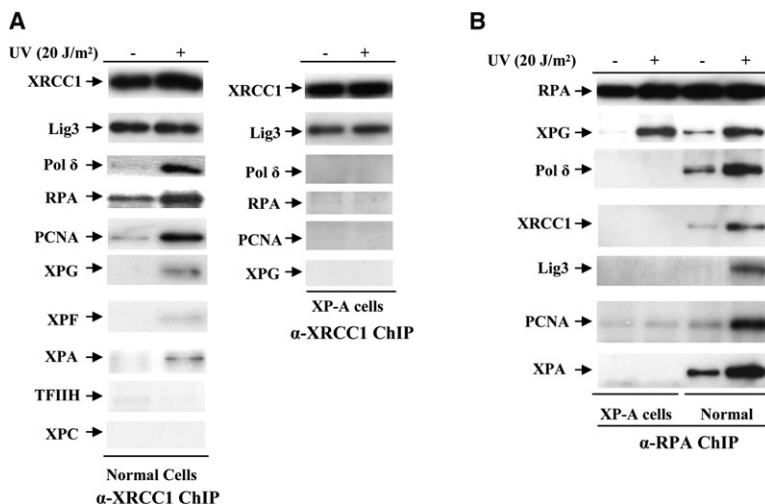
(A) Immunolocalization of PAR and p89 in normal human cells. Cells were mock treated or treated with 100  $\mu$ M H<sub>2</sub>O<sub>2</sub>, or pretreated for 30 min with 10  $\mu$ M PARP inhibitor (KU0058948) before H<sub>2</sub>O<sub>2</sub> treatment. In addition, locally UV-irradiated cells (30 J/m<sup>2</sup>) were stained for PAR and p89. (B) Immunolocalization of XRCC1 and p89 after local UV irradiation (30 J/m<sup>2</sup>) in confluent mock-treated cells or cells pretreated with KU0058948. (C) Disassembly kinetics of the NER preincision complex from UV spots in the presence of KU0058948. Disassembly was monitored by fluorescent immunostaining of p89 spots at various time points after local UV irradiation (30 J/m<sup>2</sup>) in primary human fibroblasts as indicated. Error bars represent the SEM values of 40 nuclei.

reports. No PAR spots appeared in UV-irradiated quiescent cells (Figure 3A), whereas weak and transient PAR staining occurred in a subset of cells of a UV-irradiated asynchronous population within 30 min after exposure (data not shown). Moreover, the PARP inhibitor KU0058948 (Farmer et al., 2005) had no effect on the recruitment of either p89 or XRCC1 to UV damage in quiescent normal cells (Figure 3B). The activity of KU0058948 was proven by the abolishment of PAR accumulation in H<sub>2</sub>O<sub>2</sub>-treated cells in the presence of the inhibitor (Figure 3A). Furthermore, the disassembly kinetics of the NER preincision complex did not differ significantly between UV-irradiated primary normal, Lig1-, and Lig4-deficient cells treated or mock treated with the PARP inhibitor (Figure 3C). The above results suggest that the induction of base damage or SSBs by UV irradiation must be negligible and that inhibition of PARP has no effect on the interactions of XRCC1-Lig3 with NER components at UV damage in quiescent cells.

In addition, we were unable to detect PARP-1 accumulation at UV spots in either normal or Lig1-deficient cells, whereas PARP-1 accumulation was evident at Laser (800 nm) induced DNA damage (Mari et al., 2006) (Figure S2A and data not shown). Although a role of pol $\beta$  has been implicated in NER in the presence of HU/AraC (Smith and Okumoto, 1984), recruitment of pol $\beta$  was not observed in the presence of HU/AraC (Figure S2B), in agreement with a previous report (Albertella et al., 2005). In summary, the data suggest that XRCC1 and Lig3 function in the postincision step of NER independently of PARP-1 and pol $\beta$ .

#### UV-Dependent Association of XRCC1-Lig3 with Core NER Components

The results described so far provide evidence for the involvement of two new factors, XRCC1 and Lig3, in NER. Similarly to other postincision components such as PCNA, pol $\delta$ /pol $\epsilon$ , and CAF-1 (Gillet and Schärer, 2006),



**Figure 4. UV-Dependent Association of XRCC1-Lig3 with Core NER Components**

The antibodies used for western blot analysis (WBA) are as indicated in the figure.

(A) WBA of XRCC1-specific ChIP using chromatin of crosslinked UV- and nonirradiated confluent normal and XP-A cells.

(B) WBA of RPA-specific ChIP using chromatin of crosslinked UV- and nonirradiated confluent normal and XP-A cells.

the recruitment of XRCC1-Lig3 requires dual incision of the damaged DNA (Figures 2A and 2B).

To study putative interactions between the XRCC1-Lig3 complex and NER components, we isolated NER-specific complexes by using in vivo protein crosslinking and chromatin immunoprecipitation (ChIP) (Fousteri et al., 2006). To eliminate coprecipitation of proteins involved in chromosomal DNA replication, we used nondividing cells. Cells were irradiated with a UV dose of 20 J/m<sup>2</sup>, which induces approximately four photolesions (i.e., CPD plus 6-4PP) per 10 kb of double-stranded DNA (Van Hoffen et al., 1995). After crosslinking, chromatin fragments (~300–600 bp) were precipitated with an XRCC1-specific antibody. Figure 4A shows that equal amounts of Lig3 were coprecipitated with XRCC1 from UV-irradiated and mock-treated cells. However, an association of XRCC1 with the postincision NER components RPA, PCNA, and pol $\delta$  was observed only in chromatin of UV-irradiated normal human cells. XRCC1-specific ChIP from UV-irradiated noncycling XP-A cells failed to reveal an association between XRCC1 and NER proteins, whereas the interaction with Lig3 was retained (Figure 4A). We did not detect association between XRCC1 and pol $\epsilon$  under these conditions in any of the cell lines tested. Performance of the reciprocal ChIP with an RPA-specific antibody showed increased interaction with XRCC1 and Lig3 as well as XPA and pol $\delta$  in chromatin of UV-irradiated normal cells compared to XP-A or mock-irradiated cells (Figure 4B). Nevertheless, a UV-specific association between RPA and XPG was present in chromatin of both normal and XP-A cells in agreement with previous studies (Volker et al., 2001; Riedl et al., 2003). These results provide further evidence that recruitment of XRCC1-Lig3 to UV damage is a postincision event in NER.

Western blot analysis of XRCC1-specific ChIP did not reveal any interaction between XRCC1 and the preincision NER factors XPC and p89 (TFIIH) in UV-irradiated cells (Figure 4A), in line with results from in vitro studies (Riedl et al., 2003). However, a UV-specific interaction was

observed between XRCC1, XPA, and the NER-specific endonucleases XPF and XPG (Figure 4A).

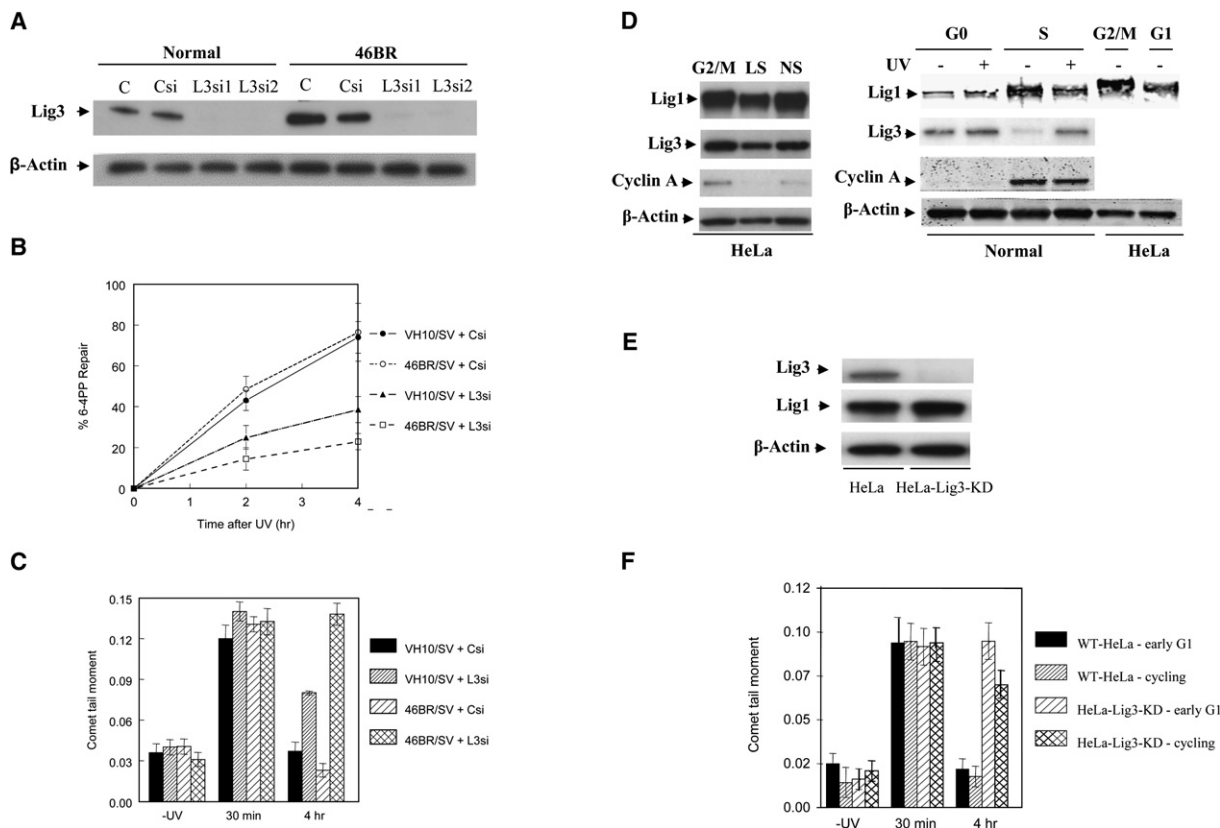
#### Downregulation of Lig3 in Human Cells Impairs Ligation of NER-Mediated DNA Breaks and Repair of UV Lesions

To test whether XRCC1-Lig3 plays an essential role in the repair of UV photolesions, we employed siRNA double-stranded oligonucleotides to knock down Lig3. To achieve efficient siRNA transfection, we employed normal (VH10/SV) and Lig1-deficient (46BR/SV) SV-40-transformed cells (Figure 5A).

Examination of 6-4PP levels in UV-irradiated Lig3si(1)-transfected normal human and Lig1-deficient cells revealed that downregulation of Lig3 significantly impaired repair of UV lesions in normal cells, and even more in Lig1-deficient cells (Figure 5B). No decrease in the repair of 6-4 PP was observed in either cell line transfected with an siRNA against GFP (control siRNA). Furthermore, the alkaline comet assay demonstrated that impaired ligation coincides with the reduced levels of repair in Lig3si(1)-transfected normal and Lig3si(1)-transfected Lig1-deficient cells (Figure 5C). These data show that, in immortalized human cells lacking both Lig1 and Lig3, repair of UV damage is severely abolished. However, the residual repair in cells lacking Lig3 only suggests that in highly proliferating cells Lig1 may partly compensate for Lig3 deficiency.

#### Cell-Cycle-Dependent Regulation of the Gap-Filling/Sealing Step in NER

Lig1 is recruited to UV spots only in S phase and late G1/S cells and might partly compensate for Lig3 deficiency in highly proliferating cells, further supporting a cell-cycle-dependent involvement of Lig1 in NER. To assess this point and to study the role of Lig3 outside S phase, we employed the alkaline comet assay and analyzed the ligation efficiency of UV-induced chromosomal breaks in synchronized HeLa cells and in HeLa cells with stable Lig3



**Figure 5. Downregulation of Lig3 Impairs the Repair of UV-Induced Lesions**

(A) WBA of normal and Lig1-deficient cells treated with a control siRNA targeted against GFP (Csi) or two different Lig3-specific siRNAs (L3si1 and L3si2).

(B) 6-4PP repair as determined by fluorescent immunostaining of 6-4PP at various times after global UV irradiation ( $15 \text{ J/m}^2$ ).

(C) Strand-break repair of globally UV-irradiated ( $15 \text{ J/m}^2$ ) cells transfected with either Csi or a L3si that were analyzed by the alkaline comet assay. Breaks were quantified as comet tail moments. Histograms are means  $\pm$  SEM of at least three independent experiments.

(D) HeLa cells were synchronized in mitosis (G2/M) by nocodazole treatment and shake off. G2/M cells were either lysed directly or replated in low (0.2%), LS) or normal serum medium (10% NS), and whole-cell extracts (WCE) were made and analyzed by WBA 6 hr after release with antibodies as indicated (left panel). The chromatin-bound fraction of UV- ( $15 \text{ J/m}^2$ ) or nonirradiated quiescent (G0) or S phase (S) VH10 htert (normal) cells was analyzed for the presence of different proteins as indicated. WCE from HeLa G2/M and G1 phase cells were included for comparison of Lig1 phosphorylation level (right panel).

(E) WCE of HeLa cells and HeLa-Lig3 KD were analyzed by western blotting. The antibodies used are as indicated.

(F) Mitotic HeLa and HeLa-Lig3 KD cells were released from nocodazole block and replated as described above for 2 hr before global UV irradiation ( $15 \text{ J/m}^2$ ). Cells were allowed to repair for 30 min or 4 hr and analyzed by alkaline comet assay. Strand break repair was quantified as comet tail moments. Histograms are means  $\pm$  SEM of at least three independent experiments.

knockdown (HeLa-Lig3-KD) (Figure 5E). We synchronized the cells in G2/mitosis (G2/M) by treatment with nocodazole and reseeded them in fresh medium containing either low (0.2%) or normal (10%) serum to delay cell-cycle progression or to allow the cells to go through G1, respectively. Two hours after release, the cells were UV irradiated and returned to the same medium to allow repair for 30 min or 4 hr at  $37^\circ\text{C}$  (the cell-cycle progression was followed by monitoring the phosphorylation status of Lig1 and cyclin A, Figure 5D) (Rossi et al., 1999). Rejoining activity was virtually absent in HeLa-Lig3-KD cells that were released in low serum (Figure 5F), indicating a complete Lig3-dependent ligation of repair patches. HeLa-Lig3-KD

cells that progressed through G1 also showed a significant ligation defect, though to a lesser extent than the noncycling cells. Full restoration of the UV-induced breaks occurred in the parental HeLa cell line, demonstrating that the synchronization procedure did not affect NER and Lig3 activity in the nocodazole-treated UV-irradiated cells (Figure 5F).

Our data support previous observations (Rossi et al., 1999; Ferrari et al., 2003) that Lig1 is inactive in G2/M phase due to CDK2-dependent hyperphosphorylation and in G0 phase due to its hypophosphorylation status (Figure 5D). UV irradiation of mitotic cells released in low serum even in the absence of a functional Lig3 did not



render Lig1 active. Interestingly, Lig3 level in the chromatin of S phase cells was substantially increased in response to UV irradiation (Figure 5D), supporting active involvement of Lig3 in repair synthesis during S phase. Taken together the above observations favor a model in which Lig3 together with XRCC1 is indispensable for the repair of UV photolesions in quiescent or postmitotic cells, whereas both XRCC1-Lig3 and Lig1 are engaged in the sealing step of NER in dividing cells.

In vitro studies have implicated pol $\epsilon$  as one of the major DNA polymerases involved in NER-dependent DNA repair synthesis. Immunolabeling of locally UV-irradiated confluent normal cells showed that pol $\epsilon$  was recruited to UV damage (Figure 6A). Using p89- or XPA-specific antibodies to monitor UV spots, we noticed, however, that similar to the picture seen for Lig1 only a subset of irradiated cells showed colocalization of pol $\epsilon$  with p89, whereas colocalization of other postincision factors such as PCNA, pol $\delta$ , and XRCC1 with XPA was evident in all cells (Figure 6C). Employing quiescent and cycling cells and immunolabeling with antibodies against pol $\epsilon$ , p89, and Ki67 (Figures 6A and 6B), we found that, parallel to Lig1, pol $\epsilon$  colocalized with UV damage only in Ki67-positive cells. Immunolabeling of UV-irradiated quiescent cells (Figure 6D) revealed accumulation of all postincision components tested except pol $\epsilon$  and Lig1 (Figures 6B and 6D). Immunolocalization of Lig1 and pol $\epsilon$  with XRCC1 in UV-irradiated confluent cells showed that accumulation of Lig1 and pol $\epsilon$  at UV-damage sites does not exclude recruitment of XRCC1 at the same sites (Figure 6E). In summary, in Ki67-positive cells, both pol $\delta$  and pol $\epsilon$  as well as XRCC1-Lig3 and Lig1 are involved in NER gap filling and ligation, whereas in quiescent cells, only pol $\delta$  and XRCC1-Lig3 are required, suggesting that NER-mediated repair synthesis and ligation in mammalian cells is differentially regulated throughout the cell cycle.

## DISCUSSION

### XRCC1 and Lig3 Are Core Components of the NER Machinery In Vivo

Efficient sealing of SSBs in chromosomal DNA during DNA replication and repair is vital in maintaining genome integrity and cell viability (Darroudi et al., 1990; Okano et al., 2000). Lig3 and XRCC1 are both required for repair of SSBs either generated during BER or induced by agents such as H<sub>2</sub>O<sub>2</sub> and camptothecin (El-Khamisy et al., 2003, 2005). XRCC1 acts as a scaffold protein to recruit, stabilize, or stimulate multiple enzymatic components of BER and SSB, including Lig3 (Caldecott, 2003).

In this study, we describe a so far unanticipated role of XRCC1-Lig3 in repair of UV-induced DNA photolesions and sealing of the chromosomal breaks in mammalian cells. In UV-irradiated cells either cycling or arrested, XRCC1 and Lig3 are recruited to sites of DNA damage. Using in vivo crosslinking and ChIP, we demonstrated that the XRCC1-Lig3 complex associates with core NER factors following UV. A number of postincision NER fac-

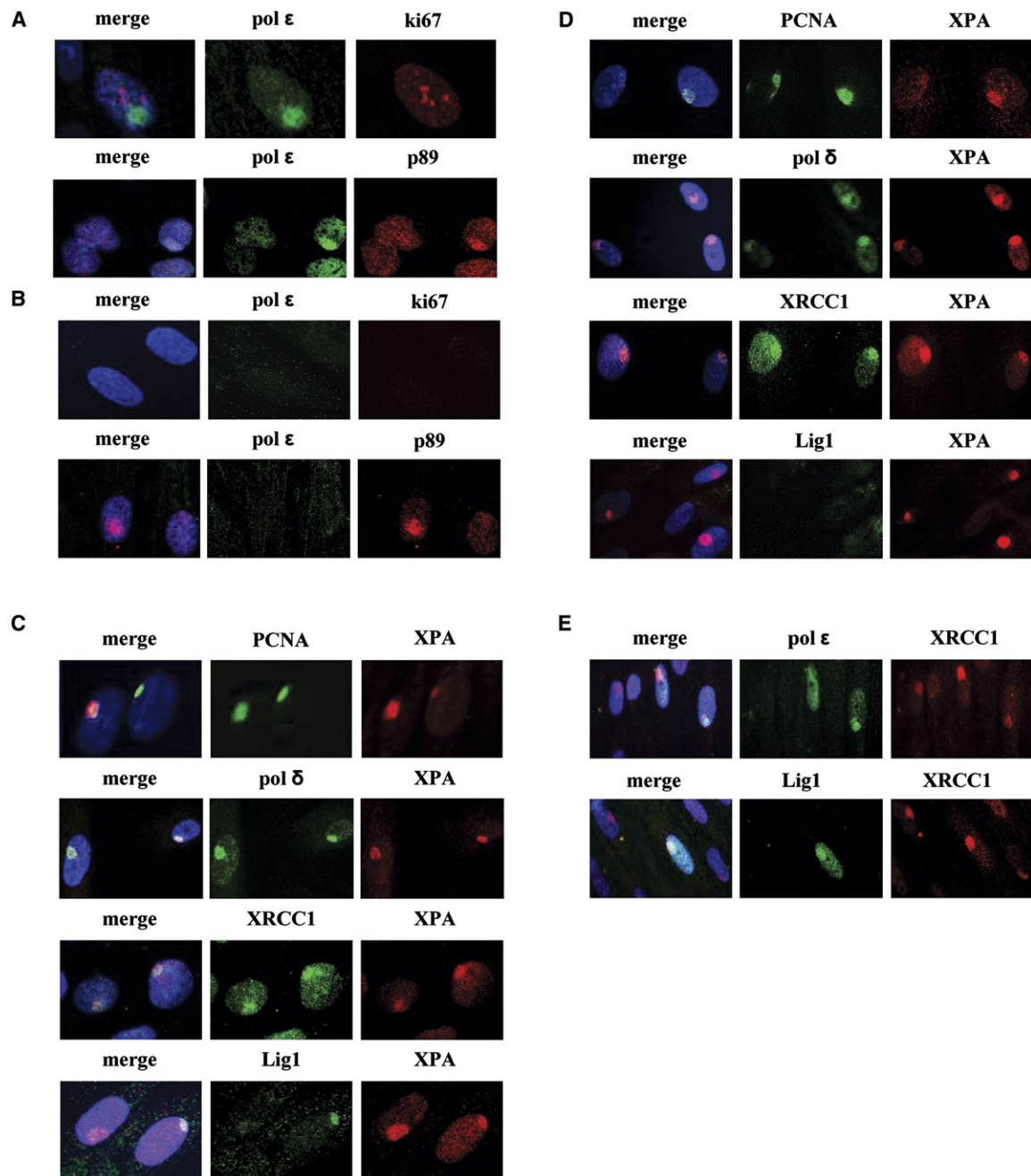
tors were detected, including PCNA, RPA, and pol $\delta$ , but surprisingly, no association with pol $\epsilon$  was found. XRCC1 has been shown to colocalize and likely to physically interact in vivo with PCNA (Fan et al., 2004). We speculate that the XRCC1-Lig3 complex might function in NER through interactions with PCNA, a key factor to recruit other post-incision NER factors. Recruitment of XRCC1-Lig3 to NER sites depends on dual incision activity, as it does not translocate to UV damage in XP-A or XP-F cells (lacking 5' incision activity). XRCC1 does not interact with the pre-incision NER components p89 and XPC in any of the ChIP reactions, although weak association with XPA, XPF-ERCC1, and XPG was found. We thus suggest that factors involved in the DNA damage recognition and the postincision steps of NER do not simultaneously reside in the same complex in vivo.

Recruitment of XRCC1 and Lig3 to SSBs in chromatin requires the activation of PARP-1 activity (Okano et al., 2003; El-Khamisy et al., 2003). Contrary to SSB, binding of PARP-1 to DNA breaks that arise during NER is not essential for the incision-dependent recruitment of XRCC1-Lig3, because no PAR signal was detected in UV-irradiated quiescent cells and the PARP-1-specific inhibitor KU0058948 had no effect on recruitment of XRCC1-Lig3 to UV damage. Obviously, the NER machinery efficiently targets XRCC1-Lig3 to sites of UV damage without the need for PAR synthesis. Taken together the results indicate that XRCC1-Lig3 plays a central role in NER: the complex accumulates at UV damage throughout the cell cycle and interacts with core NER factors in a UV-dependent manner, without the recruitment of PARP1 or pol $\beta$ . Interestingly, evidence for a positive role of XRCC1-Lig3 in a reconstituted NER system has been recently found (J.-M. Egly, personal communication).

### Downregulation of Lig3 Leads to Impaired Repair of UV Lesions and Induction of DNA Strand Breaks

Lig3 has only been identified in vertebrates and is able to take over a number of functions carried out by Lig1 in budding yeast, i.e., mitochondrial DNA replication and repair. One possibility for the involvement of Lig3 in NER in higher eukaryotes is that differentiated nondividing cells of higher eukaryotes have no active Lig1 available, and hence, repair requires specialized ligases, such as Lig3, to carry out these processes.

Lig3 is essential for early embryonic development, as targeted inactivation of the *lig3* gene in the mouse leads to embryonic lethality (Puebla-Osorio et al., 2006). However, Chinese hamster ovary (CHO) cell lines (EM9, EM-C11, and EM-C12) in which the XRCC1 protein is mutated and greatly reduced or absent have been generated (Zdzienicka et al., 1992; Caldecott et al., 1995; Shen et al., 1998). XRCC1 protects Lig3 from proteasomal-mediated degradation (Caldecott et al., 1995; Moore et al., 2000), and consequently, EM9 and EM-C11 cells exhibit 4- to 6-fold reduced levels of Lig3 protein and activity. Surprisingly, EM-C11 cells showed no sensitivity to the cytotoxic effects of UV irradiation (Zdzienicka et al., 1992). However,



**Figure 6. Cell-Cycle-Dependent Regulation of Gap Filling/Sealing in NER**

(A–E) Images were obtained with locally irradiated (30 J/m<sup>2</sup>) primary human fibroblasts.

(A) Immunolocalization of pol $\epsilon$ /ki67 and pol $\epsilon$ /p89 in cycling cells.

(B) Immunolocalization of pol $\epsilon$ /ki67 and pol $\epsilon$ /p89 in quiescent cells.

(C and D) Immunolocalization of postincision factors (PCNA, pol $\delta$ , XRCC1, and Lig1) with XPA in either confluent (C) or quiescent (D) cells. UV-damage sites are visualized by local accumulation of XPA.

(E) Immunolocalization of pol $\epsilon$  and Lig1 with XRCC1 in confluent cells.

UV-irradiated EM9 and EM-C12 cell strains exhibited a mild sensitivity in a clonogenic survival assay (Shen et al., 1998; Busch et al., 1980), and UV-irradiated EM9 cells exhibited a reduced rate of rejoining of DNA strand

breaks (Nocentini, 1999), implicating a role of XRCC1-Lig 3 in UV repair. In agreement with these results, we found reduced rejoining of UV-induced breaks in proliferating normal human fibroblasts with downregulated Lig3

and further impairment in HeLa-Lig3-KD cells synchronized in early G1 as well as in the Lig3-Lig1 double-knockdown cell line (46BR/SV + Lig3si[1]). Nevertheless, and in line with results obtained with CHO cells, clonal UV survival of Lig3si[1]-transfected normal (VH10/SV) and HeLa-Lig3-KD cells showed only mild sensitivity (data not shown). We were unable to assess UV effects in the double-knockdown cell line (46BR/SV + Lig3si[1]) because depletion of both ligases severely affected cell growth.

Inhibition of pol $\delta$ /pol $\epsilon$  in confluent normal human cells by HU/AraC leads to accumulation of DNA strand breaks and abolishment of 6-4PP repair shown in the current work and previously (Mullenders et al., 1985). Obviously, efficient gap filling by DNA synthesis and ligation of the repair patch is required to drive NER to completeness (Erixon and Ahnstrom, 1979). In accordance with this hypothesis, we found that downregulation of Lig3 in proliferating human cells leads to accumulation of DNA strand breaks and significant inhibition of 6-4PP repair, whereas Lig1 deficiency (as manifested in 46BR cells) had no impact on break frequency and repair efficiency. This finding suggests that even in S phase cells Lig3 is the dominant NER ligase. Interestingly, growing cells lacking normal levels of both Lig1 and Lig3 display synergistic impairment of NER-mediated repair. This clearly implicates a compensating role of Lig1 in 6-4PP repair in proliferating human cells with reduced level of Lig3 in spite of the apparent lack of UV sensitivity of Lig1-deficient 46BR cells.

### Lig1 Is Involved in the Repair of UV Photolesions in Late G1 and S Phase Cells

Lig1 expression levels strongly correlate with the rate of cell proliferation, consistent with its role in DNA replication (Montecucco et al., 1992; Prigent et al., 1994). Both mRNA and protein levels increase after serum stimulation of human and mouse fibroblasts, whereas confluency, serum starvation, and cell differentiation lead to a strong decrease of Lig1 gene expression (Montecucco et al., 1992). Interestingly, Lig1 activity is regulated by cell-cycle-dependent phosphorylation allowing Lig1 to be recruited to replication factories in late G1 and S phase, but not in early G1 or G2/M. In mitotic cells, Lig1 is hyperphosphorylated (at serines Ser51, Ser66, Ser76, and Ser91) and functionally inactive (Rossi et al., 1999; Ferrari et al., 2003). Although Lig1 is present in quiescent human keratinocytes, the protein is inactive and requires phosphorylation for its activation (Bhat et al., 2006). In agreement with these data, we identified hyperphosphorylated Lig1 in G2/M and hypophosphorylated Lig1 in both UV- or mock-treated quiescent cells (G0) cells. We propose that the hypophosphorylated Lig1 is unable to participate in NER, as we failed to detect recruitment of Lig1 to UV spots in quiescent cells.

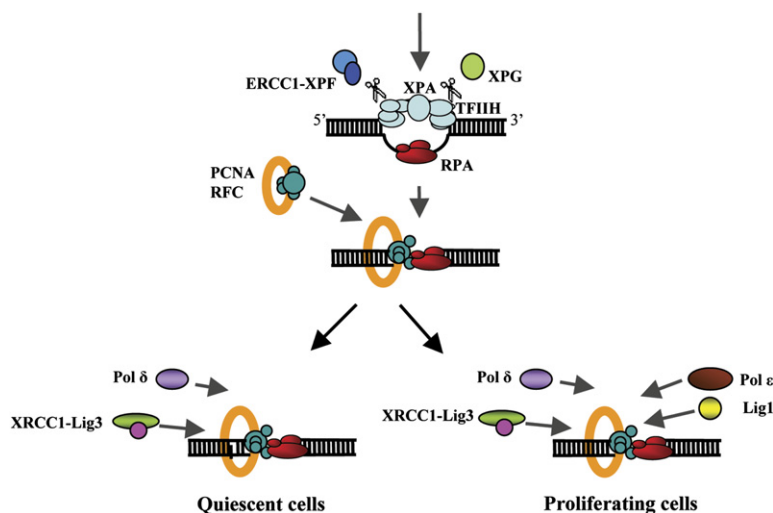
Our data are consistent with a role of active Lig1/PCNA in the ligation step of NER, but only in late G1 and S phase cells. Lig1 is recruited to UV damage in G1 and S phase cells expressing Ki67, but not in Ki67-negative G0/G1

cells. Moreover, HeLa cells with reduced levels of Lig3 were deficient in 6-4PP repair and accumulated SSBs if synchronized and irradiated in late mitosis/early G1, consistent with the presence of functionally inactive Lig1 in these cells (Ferrari et al., 2003; Rossi et al., 1999). In contrast, late G1/S HeLa cells expressing functionally active Lig1 (intermediate level of phosphorylation at Ser91 and Ser66 [Ferrari et al., 2003]) were capable of sealing UV-mediated DNA breaks, albeit less efficiently than Lig3-proficient human cells. The involvement of Lig1 in NER in S phase cells might provide an explanation for the mild UV sensitivity of Chinese hamster XRCC1 mutant cell lines that display reduced levels of Lig3 (Busch et al., 1980; Caldecott et al., 1995). It is possible that the presence of a Lig1-mediated process in rapidly proliferating cells can compensate for the absence of Lig3 over extended repair periods.

### DNA Polymerases Delta and Epsilon Work in NER via Two Distinct Complexes

Data from immunofluorescence microscopy and ChIP experiments show that pol $\delta$  and  $\epsilon$  are both involved in the repair of UV damage in vivo, consistent with a role for both polymerases in the gap-filling step of NER in vitro (Aboussekhra et al., 1995). Also, in *Saccharomyces cerevisiae*, pol $\delta$  and pol $\epsilon$  are able to carry out DNA repair synthesis in the absence of the other (Budd and Campbell, 1995). Here we show that in vivo the two DNA polymerases participate in different molecular complexes to fulfill their role in NER. ChIP experiments with chromatin from quiescent UV-irradiated NER-proficient cells revealed that the XRCC1-specific antibody pulled down exclusively pol $\delta$ , suggesting that in nondividing cells XRCC1-Lig3 predominantly associates with pol $\delta$  at sites of UV lesions. In addition, in both S phase and G0 cells, we could readily show the recruitment of XRCC1-Lig3 and pol $\delta$  to spots of UV damage, whereas we were unable to monitor accumulation of Lig1 and pol $\epsilon$  at sites of UV lesions in G0 cells.

In contrast to quiescent cells, Lig1 and pol $\epsilon$  accumulate at UV lesions in late G1 cells expressing Ki67 and in S phase cells, indicating that functionally active Lig1/pol $\epsilon$  NER complexes are formed in these cells. Earlier studies using cell-free extracts (Shivji et al., 1995) or purified components (Aboussekhra et al., 1995) revealed that pol $\epsilon$  was more suitable than pol $\delta$  for filling in short gaps in an RFC- and PCNA- dependent manner in the presence of Lig1. In addition to pol $\epsilon$  and pol $\delta$ , pol $\beta$  has been implicated in the gap-filling step of NER (Smith and Okumoto, 1984), particularly in the presence of HU/AraC. We were unable to demonstrate recruitment of pol $\beta$  to UV damage in confluent human fibroblasts in the presence of HU/AraC, and hence, it is possible that the low level of HU/AraC-resistant repair synthesis is carried out by DNA polymerase  $\kappa$  (pol $\kappa$ ). Recent evidence emerged that pol $\kappa$  implicated in bypass of replication blocks has a role in the gap-filling step in NER in UV-irradiated cells treated with HU (Ogi and Lehmann, 2006); the choice for pol $\kappa$  in the gap-filling reaction might be dependent and stimulated by the reduced dNTP



**Figure 7. Regulation of NER-Mediated DNA Repair Synthesis In Vivo**

NER postincision steps, i.e., DNA repair synthesis and ligation, and the components involved are shown. Two distinct complexes differentially carry out the gap-filling step in NER. XRCC1-Lig3 and pol $\delta$  colocalize and interact with NER components in a UV- and incision-dependent manner in dividing and non-dividing cells. In contrast, Lig1 and pol $\epsilon$  are recruited to sites of UV damage only in proliferating cells.

pools in the presence of HU. Considering that pol $\kappa$  deficiency only delays repair synthesis and that repair synthesis in confluent cells is very sensitive to pol $\delta$  and pol $\epsilon$  inhibitors such as AraC/Aphidicolin, we consider pol $\delta$  and pol $\epsilon$  as the major DNA polymerases in the gap-filling reaction of NER.

In conclusion, XRCC1-Lig3 is the dominant ligase complex involved in NER-mediated gap filling in quiescent and proliferating cells and indispensable in quiescent cells (Figure 7). Two distinct complexes differentially carry out the gap-filling step in NER. XRCC1-Lig3 and pol $\delta$  colocalize and interact with NER components in a UV- and incision-dependent manner throughout the cell cycle. In contrast, Lig1 and pol $\epsilon$  are recruited to sites of UV damage only in proliferating cells. Bulky DNA lesions such as cyclopurines are induced by reactive oxygen species generated by X-rays or endogenous cellular processes (Sato et al., 1993) and require NER for their repair (Brooks et al., 2000). Our data suggest that in quiescent or terminally differentiated cells the sealing of chromosomal DNA nicks that arise during NER would be entirely dependent on functional XRCC1-Lig3 complex. Taken together our data reveal a key role for XRCC1-Lig3 in the maintenance of genomic integrity by the NER pathway in mammalian cells and provide a possible explanation for the impact that XRCC1 polymorphic mutations have on a variety of human cancers, including sunlight-induced squamous cell carcinoma (Han et al., 2004) and lung cancer (Hao et al., 2006).

## EXPERIMENTAL PROCEDURES

### Cell Lines and Antibodies

Cell lines and antibodies used in this study are described in detail in the Supplemental Data.

### RNA Interference

Short interfering RNA (siRNA) duplexes were designed and subsequently synthesized by Invitrogen. The sequences were as follows: 5'-GCAUAAGGACUGUCUGCUA-3' to target the Lig3 transcript and 5'-GCAGACAUGCCUGACUAUU-3' for GFP targeting. A second validated siRNA against the Lig3 transcript, designed and synthesized

by QIAGEN (Cat no. S102663549), was also used. Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Repair, immunostaining, and Comet assay experiments are described in the Supplemental Data and were carried out 48 hr after transfection. The level of protein knockdown was examined by western blotting.

### In Vivo Crosslinking and ChIP

Confluent or serum-starved hTert-immortalized normal human and XP-A fibroblasts were UV irradiated (20 J/m<sup>2</sup>) and incubated at 37°C for 30 min to 1 hr prior to in vivo crosslinking (11 min, on ice) and ChIP. In vivo crosslinking, lysis of the cells, chromatin purification and immunoprecipitation, and reversal of the crosslinks prior to protein and DNA analysis were performed as described earlier (Fousteri et al., 2006).

### Comet Assay

Alkaline comet assay was performed as described in Cramers et al. (2005) with minor modifications.

### Global and Local UV Irradiation

Cells were global or locally UV irradiated through a filter with pore size of 8  $\mu$ m as described previously (Volker et al., 2001).

A more detailed version and additional experimental procedures used in this study can be found in the Supplemental Data.

### Supplemental Data

Supplemental Data include Supplemental Results, Supplemental Experimental Procedures, Supplemental References, and two figures and can be found with this article online at <http://www.molecule.org/cgi/content/full/27/2/311/DC1/>.

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